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EXECUTIVE SUMMARY OF THE THESIS

Assessment and characterization of nanovectors based on poly(2-oxazoline) for Photodynamic Therapy

TESI MAGISTRALE IN MATERIALS ENGINEERING AND NANOTECHNOLOGY – INGEGNERIA DEI MATERIALI E DELLE NANOTECNOLOGIE

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1. Introduction

In various scientific fields, medicine included, amphiphilic copolymers have attracted much attention due to their ability to self-assemble in selective solvents. Among others, polymeric self-assemblies have been studied for the last 30 years as possible nanovectors for biomedical applications, owing to their capacity to modify the biodistribution of drugs [1,2]. Uncontrolled biodistribution is a central drawback of many treatments, and the application of nanomedicine to already clinically approved techniques could improve therapeutic efficiency and reduce side effects [1].

Photodynamic therapy (PDT) is a therapeutic modality currently used for the treatment of different types of tumours, it consists in the irradiation of biological matter after inoculation of a photosensitive species (the photosensitizer), which transfers its energy to oxygen, leading to formation of reactive oxygen species. These species are toxic to the local cancerous cells. Since irradiation is a topical stimulus, PDT provides localized treatment, a highly desirable feature in the treatment of many illnesses in dermatology, ophthalmology and oncology. Although the photosensitizer is rarely specifically distributed in the area to be treated after intra-venous injection, leading to decreased efficiency.

In previous studies [3] carried out by the Laboratoire des Interactions Moléculaires et Réactivité Chimique et Photochimique (IMRCP) in Toulouse, it has been shown that encapsulating the PS in a polymeric nanovector strongly improves the PDT efficiency. The nanovectors were based on amphiphilic block copolymers functionalized with a poly(ethylene glycol) (PEG) hydrophilic block. However, studies have been increasingly showing that upon several injections of PEG- based nanovectors, an immune response occurs, induced by the production of anti-PEG antibodies, leading to a rapid clearance of the vectors [4]. In collaboration with the the Département de Chimie Moléculaire et Macromoléculaire at the Institut Charles Gerhardt Montpellier (ICGM), the IMRCP lab has begun to assess a new type of vector to be used in PDT, based on poly(2-oxazoline) [5]. In this regard the results have been encouraging suggesting that poly(2-oxazoline) could be a valid alterative to PEG in nanomedical applications.

This thesis work is a follow-up study aimed to expand this research assessing the ability of

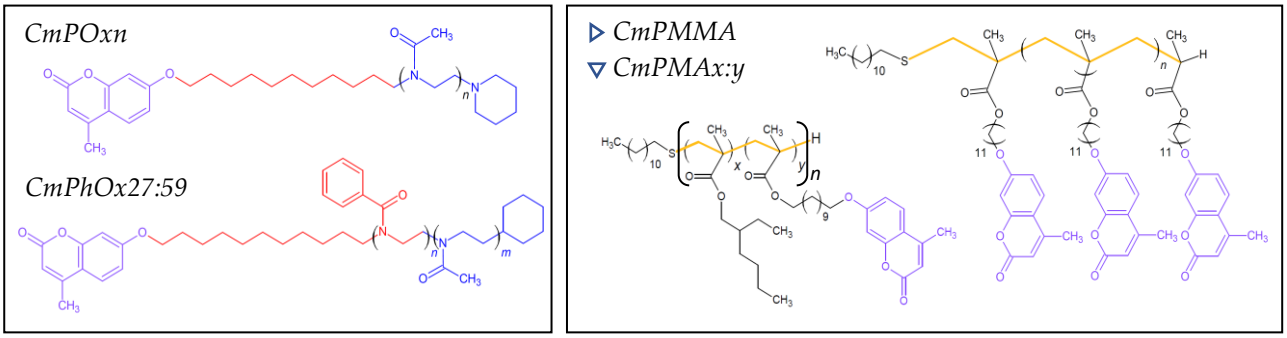

Figure 2 Chemical structure of the amphiphilic poly(2-oxazolines) (left) and of the poly(alkyl methacrylate) (right).

	Designation	Degree of polymerization n	Molecular weight [g/mol]
Coum – C_{11} – $(MOx)_{15}$	*CmPOx15*	/	1615
Coum – C_{11} – $(MOx)_{59}$	*CmPOx59*	/	5100
Coum – C_{11} – $(PhOx)_{27}$ – b – $(MOx)_{59}$	*CmPhOx27:59*	/	9700
(MA - C_{11}- Coum)$_{10}$	*CmPMMA*	/	4000
(MA - C_{11}- Coum)$_{20}$ – b – (MA - Ethylhexyl)$_{80}$	*CmPMA20:80*	27	6000
(MA - C_{11}- Coum)$_{50}$ – b – (MA - Ethylhexyl)$_{50}$	*CmPMA50:50*	20	6600
(MA - C_{11}- Coum)$_{80}$ – b – (MA - Ethylhexyl)$_{20}$	*CmPMA80:20*	18	6500

Table 2 Theoretical molecular weight and degree of polymerization of the polymers.

forming polymeric nanovectors (micelles or polymer vesicles) of a range of newly synthetised poly(2-oxazolines), considering possible influence of molecular weight and composition. Different methods for producing the nanovectors were employed varying the components to optimize the procedure. The characterization of the colloidal solutions was performed to investigate the size and morphology of the objects. Aiming to provide more stability to the vectors, UV light-induced crosslinking was also carried out thanks to the presence of photoreactive groups (coumarin) both on the chain of the amphiphilic polymers which formed the nano-vectors and on the chain of another polymer which was added to the formulation and ideally positioned itself inside the nano-object.

In collaboration with the SupraBioNanoLab (SBNLab) at Politecnico di Milano, preliminary studies on the protein corona formation on the nanovectors were carried out evaluating the stability of the obtained nanovectors in presence of biological fluid and isolating the corona-carrier complexes.

2. Materials and methods

2.1. Materials

The poly(2-oxazolines) were synthesised at the ICGM lab. Coumarin-functionalized poly(ethyl methacrylates) were provided by Specific Polymers. A poly(2-methyl oxazoline) and a coumarin- functionalized poly (methyl methacrylate) previously used in another study were also employed in the experiments. Their chemical structures are presented in Figure 1, while Table 1 summarizes their properties.

Acetone, dichloromethane, N,N-dimethylformamide, tetrahydrofuran, methanol, chloroform, sucrose, sodium chloride, potassium chloride, disodium hydrogen phosphate, potassium dihydrogenphosphate, Trizma® base, glycine, sodium dodecyl sulfate (SDS), N,N,N′,N′-tetramethylethylenediamine (Temed), ammonium persulfate (APS), Acrylamide/Bis-acrylamide (40% solution), hydrogen chloride, 2-mercaptoethanol, butanol, methanol and acetic acid – used in self-assembly formation and for protein corona analyses without further purification – were purchased from Sigma-Aldrich. For all manipulations done at IMRCP lab ultrapure water was obtained from an ELGA Purelab Flex system (resistivity higher than 18.2 MΩ cm) and was

filtered using 0.2 μm RC filters just before use. At the SBNlab ultrapure water was procured by the purification system provided by Simplicity® (18.2 MΩ cm).

2.2. Methods

Self-assemblies formation – Cosolvent method

CmPOxn – Dissolution of the polymer (8 mg/ml) in filtered water (RC cut-off 0.2 μm). *CmPhOx27:59* – Dissolution of the polymer (50 mg/ml) in acetone. Dropwise addition of the solution to 5 ml of filtered water under moderate stirring at room temperature. If needed, a small volume of additive acetone solution (10–20 μl of poly(alkyl methacrylate), calculated to have a final desired ratio of 10 wt% compared to *CmPOxn* or *CmPhOx27:59*) is added in stirring at room temperature. The solution is left standing for 2 days to evaporate acetone. Given the poor solubility of *CmPMMA* in acetone, three alternative solvents were selected to substitute acetone: dichloromethane (CH_2Cl_2), N,N-dimethylformamide (DMF) and tetrahydrofuran (THF). All the other poly(methyl methacrylates) that were employed were soluble in acetone, except for *CmPMA80:20*, which was solubilized in CH_2Cl_2 and DMF. Depending on the organic solvent, the solution was left standing for a couple of days to let the solvent evaporate or dialysis was performed on a Mini Dialysis Kit (GE Healthcare Bio-Sciences) with 1kDA cut-off and 2 ml volume.

Self-assemblies formation – Film hydration method

A 20 mg/ml polymer solution in methanol was prepared. If needed, a small volume of additive methanol/acetone solution (10–20 μl of poly(alkyl methacrylate) solution, calculated to have a final desired ratio of 10 wt% compared to *CmPOxn* or *CmPhOx27:59*) is added. The solvent was evaporated on a rotary evaporator to form a regular film which was further dried under vacuum for 4 hours. This was then rehydrated with filtered water (10 mg/ml), heated at 65 °C for 30 min and 1 h at 65 °C under sonication. The solution was then extruded on a mini-extruding system from Polar Avanti Lipids with a polycarbonate membrane (cutoff of 0.2 and 0.1 μm) on a heating plate at around 50°C. For the protein corona analysis the procedure was optimized substituting methanol with chloroform for the amphiphilic polymer and with acetone for the poly(alkyl methacrylate) and the extrusion step was soon abandoned.

Crosslinking of the self-assemblies

A 5 mm NMR tube containing 2.2 mL of the self-assembly solution was placed for 7 h between two UV lamps, Philips linear T5 8W, irradiation at 360 nm, lamp-tube distance 8 mm, total irradiance 1.0 mW/cm^2, measured using a HD9021 photometer obtained from Delta Ohm Inc.

Dynamic light scattering (DLS)

At the IMRCP lab DLS was carried out at 25 °C on a Malvern (Orsay, France) Zetasizer NanoZS with a warm-up time of 120 seconds and each measurement was the result of the average of three subsequent runs of 10 seconds each. Data were analysed using the general-purpose NNLS method and the apparent hydrodynamic radius was obtained from an intensity weighted and a number weighted fitting of the autocorrelation function. All correlograms obtained by the instrument were also analysed using a custom-made program named STORMS in order to obtain a more precise characterization of the solutions. At the SBNLab, multiangle DLS was performed on a ALV compact goniometer system at 25°C. The volume used for the analysis was between 800 μl and 1 ml. DLS was measured at different scattering angles (70°, 90°, 110°, 130°). Each measure was the result of the average of three subsequent run of 10 seconds, with a threshold sensibility of 10%. Data analysis was done with ALV-Correlator software using CONTIN algorithm and the apparent hydrodynamic radius was obtained from an intensity weighted and a number weighted fitting of the autocorrelation function.

Transmission electron microscopy (TEM) and cryogenic transmission electron microscopy (cryo-TEM)

TEM analyses were performed at the Centre de Microscopie Electronique Appliquée à la Biologie in Toulouse using a Hitachi HT7700 (Hitachi High Tech, Hitachinaka, Japan) microscope (accelerating voltage of 75 kV). Small amounts of aqueous samples were deposited onto a discharged copper grid (200 mesh) coated with a carbon membrane,

left for 1–3 min depending on the solution, and gently dried with absorbing paper. A drop of uranyl acetate solution was deposited onto the grid for 10 seconds, and the grid was then dried under a lamp for at least 5 min. At the SBNLab, TEM measurements were performed with Philips® CM200 Field Emission Gun with an electrons acceleration tension of 200 kV. The carbon coated copper grids (200 mesh) were prepared at the end of the procedure of formation of the self-assemblies, depositing 10-20 µl of solution and removing the excess with filter paper. Several images were obtained for each sample, analysing different zones of the grids. The mean size (as well as the standard deviation) was measured using the ImageJ software (200-300 nano-objects). Statistical analysis and plotting were performed using the Origin.Pro software. Cryo-TEM was performed by Dr. Stéphanie Balor (Microscopie Electronique Intégrative, Centre de Biologie Intégrative, Université Toulouse III Paul Sabatier, Toulouse, France).

Proton nuclear magnetic resonance (^{1}HNMR) spectroscopy

^{1}HNMR spectra were used to verify the absence of residual solvent in the aqueous solutions prepared with cosolvent method and to ascertain the occurrence of dimerization of the coumarin groups on the polymeric chains. Samples were freeze-dried, dissolved in about 1 ml of deuterated chloroform, and inserted in 5mm NMR tubes; in same cases the sample were directly prepared in deuterated water to be directly analysed without drying. NMR spectra were recorded on 300 or 400 MHz Bruker spectrometers and consequently analysed with Bruker TopSpin software in France and MestreNova in Italy.

Protein corona analysis – centrifugation, gel electrophoresis, and stability analysis

Our samples were diluted 1:2 (final concentration 1.25 mg/ml) in filtered phosphate buffered saline (PBS) (RC 0.2 µm) with 10% of fetal bovine serum (FBS), then incubated for 1 hour at 37°C and 95% relative humidity (RH). They were added to a 0.7M sucrose cushion solution, which was then followed by centrifugation for 30 minutes at 18000×g and 4°C and washing with filtered PBS; centrifugation and washing were both repeated 3 times. DLS analysis was performed each time on the resuspended sample and the supernatant solution to verify the presence of NPs complexes. Ultracentrifugation with sucrose gradient was performed by Dr. Beatrice Lucia Bona at the laboratories of IRCCS (Institutes for comprehensive cancer patient care and research) Humanitas Cancer Center. Centrifugation polypropylene tubes (Beckman Coulter) of 13 ml were prepared layering 1 ml solutions of sucrose in water from the most to the less concentrated one; starting from a stock sucrose solution of 30%wt, other 10 solutions were obtained through different dilutions; the tube was left to equilibrate overnight to create a continuum gradient. Before centrifugation, 0.7 ml of our sample was added on the top of the gradient. Ultracentrifugation was performed for 1 hour at 60000×g and 20°C. Aliquots of 1 ml each were carefully collected from the top to the bottom of the tube. Dialysis was performed overnight at 4°C on all the layers with pieces of Spectra/Por® 6 Dialysis Membrane, pre-wetted RC tubing (MWCO 2 kD) closed with zip-ties to remove sucrose.

Electrophoresis was performed on a BioRad Mini-PROTEAN® Tetra system following a pre-existing protocol. A Precision Plus ProteinTM Standard All Blue purchased from BioRad was used as protein marker. The samples were concentrated from 1 ml to 20 µl using Corning® Spin-X® UF concentrators, 100K MWCO purchased from Sigma-Aldrich. The gels were consequently stained with 2D-Silver Stain Reagent II purchased from Cosmo Bio Co.,Ltd following the procedure provided by the company.

Stability of the objects was evaluated by DLS analysis registering the change of the hydrodynamic radius of the NPs in a solution of PBS (dilution 1:2) in presence of 10%FBS. After the incubation step, DLS analysis was performed after 1 hour, 4 hours and 24 hours maintaining the sample at 37°C and RH = 95%.

3. Results and discussion

3.1. Results

The polymeric nanovectors are composed of a hydrophilic stabilizing external block, the poly(2-methyl oxazoline) and a hydrophobic core, the C11 alkyl chain ($Coum - C_{11} - (MOx)_n$). In the second type of synthesised copolymer ($Coum - C_{11} - (PhOx)_{27} - b - (MOx)_{59}$) the hydrophobic section

was lengthened by the addition of a phenyloxazoline block, incorporating bulky aromatic rings in the polymer chains. All these copolymers presented the photoreactive moiety coumarin in their structure, which dimerizes upon UV irradiation. Following a procedure of previous studies, coumarin-functionalized poly(alkyl methacrylates) ensured stabilization by crosslinking by photo-dimerization both with their own coumarin unit and the ones of the amphiphilic copolymers.

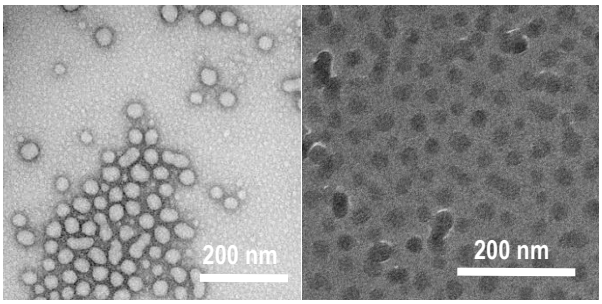

Figure 2 TEM (left) and cryo-TEM (right) images of *CmPhOx27/59* nanovectors prepared with film rehydration method.

Characterization of the self-assemblies

CmPOx was directly dispersed in water and small 10 nm nano-objects were observed *via* TEM. Similar objects were present in the systems where a stabilizing poly(alkyl methacrylate) was added using an acetone solution, except in the case of *CmPMA50:50* where the size of the self-assemblies was around 30 nm. Varying the solvent had a noticeable influence on the systems, especially in the case of DMF, since a second population almost an order of magnitude larger than the other was observed. Although TEM makes possible to visualize the self-assemblies, it has well-known limitations due to the drying procedure. Therefore, Dynamic Light Scattering (DLS) measurements were also performed enabling to determine a size distribution of the sample dispersion. The intensity-average analysis showed the presence of large objects, with a population above 300 nm. This size, however, varied greatly with the solvent and the choice of poly(alkyl methacrylate). On the other hand, number average analysis showed that the more dominant population was a smaller one (with size of about 20 nm) in agreement with TEM results. However, the presence of the larger population, even if less dominant cannot be ruled out.

Using film rehydration method, small nano-objects around 10 nm were observed in TEM images, together with a second larger population which was clearly present only for the systems composed by *CmPOx* alone. The intensity-average analysis showed the presence of large nano-particles (up to 1 µm), the nature of which was not investigated, further analyses with a variation in the concentration of the polymer could be useful in this regard.

Being not soluble in water, *CmPhOx27:59* was solubilized in acetone (or alternatively in CH_2Cl_2 or DMF) and then added dropwise to water, alone or together with a stabilizing poly(alkyl methacrylate). Two populations were observed *via* TEM (Figure 2 and Table 2), one around 10 nm and the second around 30 nm and a much wider distribution. The addition of *CmPMMA* did not

	DLS Int	DLS Num	TEM	Cryo-TEM
CmPhOx27:59	123	71	11 ± 3/29 ± 10	40 ± 17
CmPhOx27:59/ CmPMMA	209	115	10 ± 2/29 ± 7	28 ± 23
CmPhOx27:59/ CmPMA20:80	228	114	27 ± 6/56 ± 15	-
CmPhOx27:59/ CmPMA50:50	252	115	23 ± 6	-

Table 2 Characterization of polymer self-assemblies prepared with acetone cosolvent method.

	DLS Int	DLS Num	TEM	Cryo-TEM
CmPhOx27:59	145	82	10 ± 2/34 ± 5	27 ± 4
CmPhOx27:59/ CmPMMA	162	89	11 ± 2/34 ± 4	-
CmPhOx27:59/ CmPMA80:20	3911	10	11 ± 2/30 ± 4	-

Table 3 Characterization of polymer self-assemblies prepared with film rehydration method.

seem to affect the results, while bigger populations were detected using *CmPMA20:80* and *CmPMA50:50*. These stabilizing polymers seemed to also affect the morphology of the systems since worm-like objects were detected *via* TEM. Cryo-TEM characterization showed a wide and diverse distribution of nano-objects with dimensions similar to the population of larger size present in TEM images. The results were less sensitive to the variation of solvent in the procedure. This was verified also in DLS analysis, which reported an intensity-average size around 200 nm and a number-average size around or lower than 100 nm, again underlining the discrepancy between TEM and DLS results, however, cryo-TEM appears to confirm the values obtained with number-average analysis.

For the copolymer bearing phenyloxazoline units, both TEM images and DLS analyses on the solutions prepared with film rehydration method were consistent with results obtained with acetone cosolvent method (Figure 2 and Table 3). Cryo-TEM confirmed the presence of only one population similar to the one of larger size in TEM, in this case, however, its size distribution was much narrower.

Crosslinking of the self-assemblies

Crosslinking was performed on *CmPOx15/CmPMMA* and *CmPhOx27:59/CmPMA50:50* self-assemblies. The occurred dimerization of coumarin was confirmed by the right shift of the peaks of interest in the ^{1}HNMR spectrum. The nano-objects formed by *CmPOx15/CmPMMA* were characterized with DLS and TEM and the results were coherent with the ones obtained in the previous study, showing that neither size distribution nor shape of the self-assemblies were modified upon crosslinking.

Crosslinking of *CmPhOx27:59/CmPMA50:50* systems was carried out at the IMRCP lab in Toulouse by Dr. Orélia Cerlati requiring at least 12 hours for a complete dimerization. Reproducing of the process at the SBNLab in Milan was attempted with the use of a photoreactor, employing the same type of lamps but with the distance between the sample and the lamps increased to 3 cm. This attempt failed since crosslinking was not observed even after 14 hours. No further attempts were made.

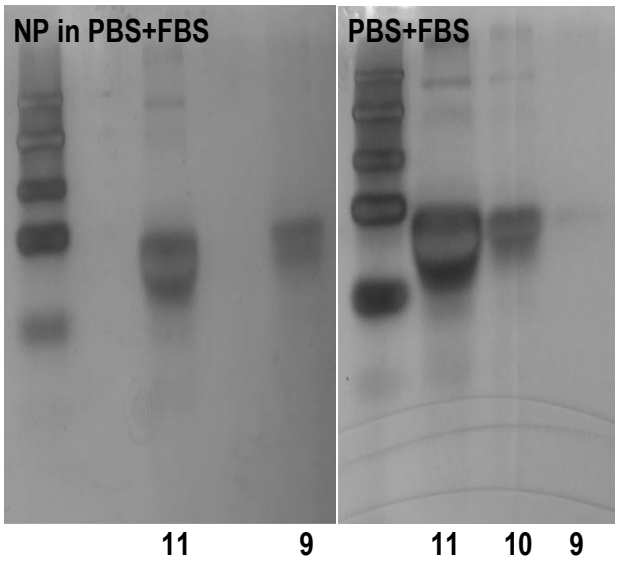

Figure 3 Comparison of SDS-PAGE gel of the layers of NP solution diluted with PBS+FBS (left) and of PBS+FBS only (right).

Protein corona analysis

CmPhOx27:59 was selected to perform protein corona analysis together with one of the stabilizing poly(alkyl methacrylates), since its characterization results showed narrower populations with lower polydispersity with respect to the other polymer. Moreover, the polymeric systems seemed less sensitive to the choice of formation method and stabilizing polymer. To avoid solubility issues, the protein corona analyses were carried out only on self-assemblies prepared with the film rehydration method. The final concentration of the solution was decreased to 2.5 mg/ml and an optimized procedure was implemented.

Both non-crosslinked and crosslinked samples were characterized after incubation in presence of biological fluid (FBS) and no change in size was observed suggesting the lack of corona formation and a confirmation of the biofouling properties of poly(2-oxazolines) [6].

Stability analysis showed no change in the size of both non-crosslinked and crosslinked systems in the 24 hours of the analysis. For this reason, isolation of the protein corona–carrier complexes was performed on systems formed only by *CmPhOx27:59*.

Sucrose cushion centrifugation was unsuccessful in isolating the complexes since nano-object were still observed in the supernatant solution after three cycles. Ultracentrifugation with sucrose gradient showed different results for the samples

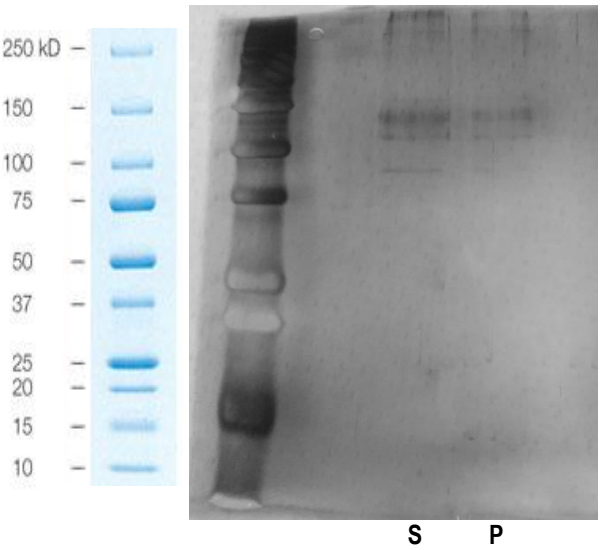

Figure 4 SDS-PAGE gel of resuspended pellet solution (P) and supernatant solution (S) after centrifugation.

containing the NPs and the control ones confirming the isolation of the complexes from the biological fluid. DLS analysis was only partially possible due to unexpected leakage of the samples from the membrane tubes, however, systems around 60 nm and around 200 nm were observed in the layers of the NP sample. Gel electrophoresis of the samples obtained from ultracentrifugation seemed to confirm the occurred isolation of the corona-carrier complexes in one of the less concentrated layers (Figure 3). Moreover, SDS-PAGE results on the samples from both centrifugation and ultracentrifugation showed very few protein bands (Figure 3 and 4), again confirming the biofouling properties of poly(2-oxazoline)-based nanovectors.

3.2. Discussion

Developing polymeric nanovectors implies characterization of the obtained self-assemblies, which can often be challenging, owing to possible co-existence of multi-populations. The developed nanovectors in this thesis work have indeed shown the presence of multi-population. Both TEM and cryo-TEM indicated the presence of relatively small nano-objects below 40 nm, whereas intensity-average DLS analysis showed the presence of also larger agglomerates ranging from 200 nm to 1 μm depending on the sample. This discrepancy can be explained by the intrinsic weakness of DLS in analysing multi-population systems and was previously observed when studying poly(2-oxazoline) systems [5]. Moreover, a number weighted analysis suggests that the larger population is the less dominant. Developing an appropriate method to characterize the self-assemblies was beyond the scope of this work, but should be performed to get a precise knowledge of the vector solution content. Among the possible approaches, Nanoparticle Tracking Analysis (NTA) is a technique often used to characterize vesicles, providing both their size and the distribution of different populations. Another one is Flow Field Flow Fractionation (F-FFF), providing a separation of the nano-objects by their size before analysis by light scattering.

In this work, we selected the most promising sample and we studied their behaviour in a biological environment before and after cross-linking. Given the low density of the polymer, it is difficult to isolate these NPs from the excess protein. We attempted to isolate protein-corona-NP complexes using a simple sucrose cushion centrifugation approach, but it was not efficient as we lost a large number of NPs in the washing supernatant. Thus, we tried to isolate the protein-corona-NP complexes by ultracentrifugation in sucrose gradient. This approach allowed the isolation and recover of the NP from the protein excess. SDS-PAGE results did not show protein bands characteristics of a protein corona, indicating good biofouling properties. Unluckily, DLS analysis did not give conclusive results and should be repeated. Further analyses are required to confirm unambiguously the biofouling behaviour of poly(2-oxazoline) vectors in biological fluid and compare it with PEG-based carriers.

The stability of the systems should be investigated further increasing the percentage of biological fluid. Indeed, the results suggests that the systems formed by *CmPhOx27:59* remain unchanged for 24 hours even without crosslinking. This could be a turning point in the study since it could lead to a focus on the tailoring the polymeric chain structure of the poly(2-oxazoline) to verify if comparable or better results in PDT efficiency are possible with respect to the ones obtained with crosslinked vectors.

4. Conclusions

The ability of forming polymeric nanovectors of a range of newly synthetised poly(2-oxazolines) was assessed and the objects were characterized

confirming the self-assembly of possible carriers for nanomedicine applications, in particular PDT. Tuning the structure of the polymer is a promising mean to design optimized nanovectors. Further work is needed to confirm the biofouling character of poly(2-oxazolines) in biological environment.

5. Acknowledgements

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